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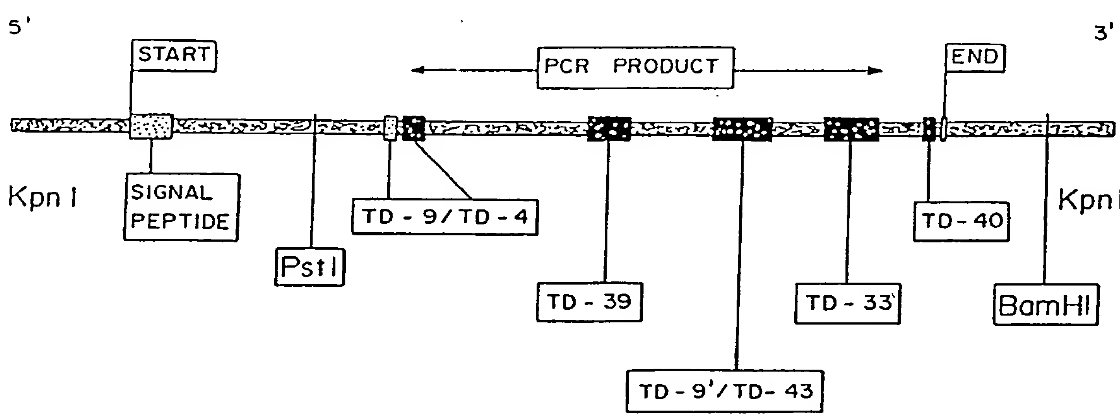
(54) Title: HEPARINASE GENE FROM *FLAVOBACTERIUM HEPARINUM*



(57) Abstract

The cloning of the heparinase gene from *Flavobacterium Heparinum* using the polymerase chain reaction is described. The Open Reading Frame (ORF) corresponded to 1152 base pairs encoding a precursor protein of MW 43,800 daltons. The amino acid sequence reveals a 20-residue leader peptide. The gene was expressed in two expression systems in *E. Coli*.

HEPARINASE GENE FROM *FLAVOBACTERIUM HEPARINUM*

Background of the Invention

This invention is generally in the area of heparinases and is specifically directed to the gene encoding heparinase I, expressed in Flavobacterium heparinum.

The United States government has rights in this invention by virtue of grant number 25810 from the National Institutes of Health.

Heparin is an anticoagulant that activates serine protease inhibitors (serpins), which play a key role in the blood clotting cascade, as described by Damus et al., *Nature* 246:355-357 (1973). According to Lindahl et al., *Trends Biochem. Sci.* 11:221-225 (1986), heparin is the most acidic natural polymer known to date. It consists of a major 1,4-linked disaccharide repeating unit of D-uronic acid 1,4-ß-D-glucosamine, and has an average of four negative charges (three sulfate groups and one carboxylate group) per monosaccharide unit. Heparin is both polydisperse, having an average molecular weight between 3,000 and 45,000 daltons, and heterogenous due to partial epimerization of D-glucuronic acid to L-iduronic acid and incomplete N- and O- sulfation, as reported by Kusche et al., *Proc. Natl. Acad. Sci.*, 77:6551-6555 (1980) and Comper, *Polymer Monograph* 7, 1981.

In addition, proteoglycans like heparin have a wide range of biological influences, including in blood chemistry, growth factor interaction and wound healing, interaction with basic structural proteins in the extracellular matrix and in cellular mediated immune responses. The basic nature of protein/peptide heparin/complex carbohydrate interaction is important. Although heparin seems fairly heterogenous, it is now quite clear that different heparin fractions exhibit distinct and unique properties indicating some

activation energy of 4.5 kcal/mol, a km of 8 x 10-6 and a Vmax of 4 x 10-7 M/min.

Heparin is often used in surgery to prevent blood clotting and to increase the compatibility of extracorporeal devices such 30 as heart-lung and kidney dialysis machines. The enzymatic degradation of heparin by heparinase is sufficient to eliminate the anticoagulation properties of heparin in surgery. As described by Langer, et al. in *Biomaterials: Inter-facial Phenomenon and Applications, Adv. in Chem. Symposium Series*, Chap. 13, pp. 493-509 (1982), this property has led to the use of heparinase as an immobilized bioreactor in conjunction with heart-lung or kidney dialysis machines to deheparinize blood. Commercial application of the heparinase bioreactor is pending clinical trials.

A principal problem in the use of the heparinase bioreactor is the availability of sufficient amounts of pure heparinase to be immobilized onto a surface. This is primarily because the amount of heparinase constitutively expressed in *F. heparinum* is very low. Inducing expression of heparinase in *F. heparinum* with heparin is very expensive due to the amounts of heparin needed and the size of the fermentation to produce reasonable amounts of heparinase for any practical applications.

Cloning and expression of the heparinase gene is important in several ways. First, the only enzyme cloned and characterized to date which acts to depolymerise proteoglycans is heparinase. Second, heparin is the only anticoagulant commonly used in surgery so deheparinizing blood is an important medical problem. Moreover, heparinase catalyzed degradation of heparin into lower molecular weight heparin molecules can be used to yield products with specific anticoagulant activity, as discussed by

Summary of the Invention

The cloning of the heparinase gene from *Flavobacterium Heparinum* using the polymerase chain reaction is described. Two degenerate oligonucleotides, based on amino acid sequence derived from tryptic peptides of purified heparinase were used in the PCR with *Flavobacterium* genomic DNA as the template to generate a 600 base pairs probe. This probe was used to screen a pUC 18 *Flavobacterium* genomic library. The Open Reading Frame (ORF) corresponded to 1152 base pairs encoding a precursor protein of MW 43,800 daltons. Eleven different tryptic peptides (approximately 48% of the total amino acids) mapped into the ORF. The amino acid sequence reveals a 20-residue leader peptide.

Heparinase can be expressed from the gene. Additionally, the gene can be modified to produce heparinase with altered enzymatic activity, specificity, or binding properties. The sequence can also be used as a probe in the isolation of genes encoding other related enzymes.

Brief Description of the Drawings

Figure 1 is a schematic representation of the PCR products Y1:C and D:C which are 600 and 160 basepairs, respectively. The 600 basepair PCR product was used as a template with D and C as primers to generate the 160 basepair D:C product.

Figure 2 is the restriction map of the genomic DNA pUC 18 plasmid, pRS.HEP51, having an insert containing the heparinase gene. The plasmid is 5631 bases long and has approximately 2300 bases of insert. The heparinase gene is in the *Kpn I-KpnI* fragment.

Figure 3 is a *Kpn*I-*Kpn*I fragment map showing the heparinase gene structure with the different tryptic peptides mapping into the open reading frame. Six

AAA ATG CCC TTT GCC CAG TTC CCT AAA GAT TGC TGG ATT
ACT TTT GAT GTC GCC ATA GAC TGG ACG AAA TAT GGA AAA
GAG GCC AAT ACA ATT TTG AAA CCC GGT AAG CTG GAT GTG
ATG ATG ACT TAT ACC AAG AAT AAG AAA CCA CAA AAA GCG
CAT ATC GTA AAC CAG CAG GAA ATC CTG ATC GGA CGT AAC
GAT GAC GAT GGC TAT TAC TTC AAA TTT GGA ATT TAC AGG
GTC GGT AAC AGC ACG GTC CCG GTT ACT TAT AAC CTG AGC
GGG TAC AGC GAA ACT GCC AGA TAG (stop codon)

The following is the amino acid sequence (Sequence No. 2) of heparinase:

Met Lys Lys Gln Ile Leu Tyr Leu Ile Val Leu Gln Gln
Leu Phe Leu Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser
Gly Asn Ile Pro Tyr Arg Val Asn Val Gln Ala Asp Ser
Ala Lys Gln Lys Ala Ile Ile Asp Asn Lys Trp Val Ala
Val Gly Ile Asn Lys Pro Tyr Ala Leu Gln Tyr Asp Asp
Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr Arg Phe Glu
Leu Lys Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala Ala
Gly Glu Thr Lys Gly Arg Thr Glu Leu Ser Tyr Ser Tyr
Ala Thr Thr Asn Asp Phe Lys Lys Phe Pro Pro Ser Val
Tyr Gln Asn Ala Gln Lys Leu Lys Thr Val Tyr His Tyr
Gly Lys Gly Ile Cys Glu Gln Gly Ser Ser Arg Ser Tyr
Thr Phe Ser Val Tyr Ile Pro Ser Ser Phe Pro Asp Asn
Ala Thr Thr Ile Phe Ala Gln Trp His Gly Ala Pro Ser
Arg Thr Leu Val Ala Thr Pro Glu Gly Glu Ile Lys Thr
Leu Ser Ile Glu Glu Phe Leu Ala Leu Tyr Asp Arg Met
Ile Phe Lys Lys Asn Ile Ala His Asp Lys Val Glu Lys
Lys Asp Lys Asp Gly Lys Ile Thr Tyr Val Ala Gly Lys
Pro Asn Gly Trp Lys Val Glu Gln Gly Gly Tyr Pro Thr
Leu Ala Phe Gly Phe Ser Lys Gly Tyr Phe Tyr Ile Lys
Ala Asn Ser Asp Arg Gln Trp Leu Thr Asp Lys Ala Asp
Arg Asn Asn Ala Asn Pro Glu Asn Ser Glu Val Met Lys
Pro Tyr Ser Ser Glu Tyr Lys Thr Ser Thr Ile Ala Tyr
Lys Met Pro Phe Ala Gln Phe Pro Lys Asp Cys Trp Ile
Thr Phe Asp Val Ala Ile Asp Trp Thr Lys Tyr Gly Lys
Glu Ala Asn Thr Ile Leu Lys Pro Gly Lys Leu Asp Val
Met Met Thr Tyr Thr Lys Asn Lys Lys Pro Gln Lys Ala

for 24 hour. The reaction was terminated by heating the sample at 65°C for 2 minutes. The digest was separated by reverse phase HPLC using a gradient of 0 to 80% acetonitrile. The tryptic peptides were monitored at 210 and 277 nm.

The tryptic peaks were collected in Eppendorff tubes. Based on the homogeneity of the peptide peak, eight different peaks were sequenced using an Applied Biosystems sequencer, model 477, with an on-line model 120 PTH amino acid analyzer located in the Biopolymers lab, Center for Cancer Research, MIT. The sequences are set forth in Table I below. The designation (K,R) is used in Table I to indicate that trypsin cuts at either lysine or arginine residues. The asterisks in Table I represent amino acids that could not be determined. The peptide designated td Lx is the longest peptide sequenced having 38 residues. Native heparinase was also sequenced to determine the N-terminus amino acids.

Table I: **Sequences of Tryptic Peptides of Heparinase**

Peptide	Amino Acid Sequence
td 04	(K, R) G I C E Q G S S R
td 09	(K, R) T V Y H Y G K
td 09′	(K, R) T S T I A Y K
td 21	(K, R) F G I Y R
td 33	(K, R) A D I V N Q Q E I L I G R D D * G Y Y F K
td 39	(K, R) I T Y V A G K P N G N K V E Q G G Y P T L A F *
td 43	(K, R) M P F A Q F P K D C W I T F D V A I D * T K
td 40	(K, R) N L S G Y S E T A R
tdm4	K N I A H D K V E K K
td 72	K T L S I E E F L A L Y D R
td Lx	R S Y T F S V Y I P S S F P D N A T T I F A W H G A P S R T L V T P E I K

primers:

A 5′- ATI AA(T/C) CA(A/G) GA(A/G)ATI (C/T)TI AT(T/C/A) GG -3′

B 5′- CCIATIA(G/A) IAT (T/C)TC (T/C)TG (T/C)TG (A/G)TT ICA (A/C)AT

C 5′- CCIATIA(G/A) IAT (T/C)TC (T/CTG (T/C)TG (A/G)TT ICA (T/G)AT -31

Of the six RHPLC peaks initially sequenced (Table I), three were chosen for primer design. Three sets of primers were designed (Table II). The PCR product of the combination the primers td43 and td33 was about 150 base pairs in length. The combination of td4 and td33 primers were about 600 base pairs. Primer td43 was 5′ to primer td33 and primer td4 was 5′ to td43 primer. Using the PCR product of td4 and td33 as a template and td43 and td4 as primers the predicted 150 base pair product was obtained confirming that td43 was between td4 and td33.

The 600 basepair product shown in Figure 1 represents about 51% of the approximated total 1170 base pairs for the heparinase gene, assuming 43,000 dalton for heparinase and a 110 dalton average amino acid with a molecular weight corresponding to about 390 amino acids times three which is 1170 bases.

The 600 base pair probe was chosen for screening a pUC 18 library by high stringency colony hybridization. Two positive clones were identified which were carried through for three rounds of colony purification.

Genomic DNA, RNA, and Plasmid Library

The *F. heparinum* genomic DNA was isolated by the A.S.A.P.™ kit (Boehringer Mannheim, Indianapolis, IN) with the following modifications. The DNA was desalted over a Sephadex™ G-50 column (Nick column, Pharmacia, Piscataway, NJ) and concentrated using a Centricon™ P-30 (Amicon Division, Beverly, MA) to a final volume of 100 1. From 1 x 10^9 cells, 105-115 g of DNA typically were obtained. Total cellular mRNA was isolated using the

the PCR product run was isolated from a low melt agarose gel, denatured by boiling at 95°C for 10 minutes, and then chilled on ice. To the denatured DNA were added 10 mM dNTPs (dATP, dGTP, dCTP, dTTP), random hexanucleotides in the reaction buffer, and 50 μCi of ^{32}PdCTP(3000 Ci/mmole). The reaction was carried with Klenow for 30 minutes at 37°C and terminated using 0.2 M EDTA. Following the labelling reaction, the labelled probe was purified from the free nucleotide by using a Sephadex G-50 column (Nick Column, Pharmacia, Piscataway, NJ). The colonies were screened with the labelled probe using standard colony hybridization procedures as described by Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, incorporated herein by reference.

Two positive clones were isolated and the plasmids tested for their ability to generate the 600 basepair PCR product. Both of the clones tested positive and were further characterized by restriction mapping. Clone pRS Hep 51 is a 2.3 kb insert in pUC 18 (shown in Figure 2) with a *Kpn-Kpn* fragment of about 1.6 kb. This fragment was a positive template for generating a 600 basepair PCR product. The *Kpn*I-*Kpn*I fragment of pRS 51 was subcloned into M13 and sequenced.

<u>DNA Sequencing</u>

DNA sequencing was performed using phage M13 and employing the dideoxyadenosine 5'-alpha-^{35}S-triphosphate and Sequenase (US Biochemical Corp, Cleveland, OH) as described by the manufacturer. The sequence data was obtained using successive nested deletions in M13 using T4 DNA polymerase as per Cyclone I Biosystems (International Biotechnologies Inc., New Haven, CT) or sequenced using synthetic oligonucleotide primers.

The sequence reveals a single, continuous open reading frame (ORF) of 1152 basepairs corresponding to

the Omp A pIN vector with the *E. coli* periplasmic leader sequence. DH5α was transformed and expression was induced with 1 mM IPTG for 3-5 hours.

As shown in Table III, the construct of the Omp A expression system results in two extra amino acids at the amino terminal of the heparinase gene, Gly and Ile. The heparinase sequence begins with a Gln.

The pKK expression system

The pKK expression system is used for over-expression of proteins in accordance with the methods of Brosius and Holy, *Proc. Natl. Acad. Sci.*, 81: 6929 (1984) and Jaffe et al. , *Biochem.* 27:1869 (1988), incorporated by reference herein. This system contains a strong *tac* promotor which, in appropriate hosts, is regulated by the *lac* repressor and induced by the addition of IPTG, as in the Omp A system. The plasmid pKK223-3 has a pUC 8 multiple cloning site and a strong rrnB ribosomal terminator immediately following the *tac* promotor. The ribosomal binding site of the plasmid was utilized by cloning the heparinase gene into a *Sma*I site, which is about 12 bases from the start codon ATG. Like the Omp A construction, the heparinase insert is obtained by PCR with *Sma*I and *Hind*III restriction sites at the N and the C terminals of the protein. As shown in Table III, the native heparinase leader sequence was used for over-production into the periplasm.

Periplasmic proteins of *E. coli* were isolated by osmotic shock. Briefly, 1.5 ml of cells were centrifuged after induction and washed with 10 mM Tris pH 7.5. The cells were then suspended in 20% sucrose in 10 mM Tris pH 7.5 and 5 μl of 0.5 M EDTA. After a five minute incubation on ice, the cells were centrifuged and osmotically shocked by adding approximately 150 μl water. The periplasmic extract was used to determine enzyme activity. Heparinase activity was determined by monitoring the wavelength at 232 nm and by the Azure A

Table III: Design of OmpA and pKK plasmids for expression of Recombinant Heparinase in *E.coli*

Omp A secretion Expression system

N	Gly	Ile	Gln	Lys		Thr	Ala	Arg	End	C
XXX	**GGA**	**ATT**	CAG	AAA	---------	ACT	GCC	AGA	TAG	**GGATCC**XXX
XXX	**CCT**	**TAA**	**GTC**	TTT	---------	TGA	CTT	ACT	ATC	**CCTAGG**XXX
	EcoRI								*Bam* HI	

pKK over-Expression system

N	Met	Lys	Lys				Ala	Arg	End						
XXX	Taa	**CCC**	**GGG**	ATG	AAA	AAA	-----	GCC	AGA	TAG	**AAG**	**CTT**	CCG	XXX	
XXX	ATT	**GGC**	**CCC**	TAC	TTT	TTT	-----	CGG	TCT	ATC	**TTC**	**GAA**	GGC	XXX	
		Sma I													*Hind* III

of the peak profiles and some peaks which were isolated and sequenced.

A positive signal was obtained for the isolated *F. heparinum* mRNA using the 600 basepair probe derived from the PCR which has been used for isolating the heparinase gene, confirming that the gene isolated was a *F. heparinum* gene cloned in *E. coli*.

The expressed heparinase appeared to have at least some heparinase activity.

The sequence can be modified to alter specific enzymatic activity or binding specificity or affinity by substitution of one or more amino acids, using site directed mutagenesis or substitution of oligomers into the sequence encoding the heparinase. Methods and materials to accomplish this are known to those skilled in the art. The modified gene is then expressed and the product routinely screened for the altered activity.

Although described with reference to two specific expression systems, other expression systems are well known and commercially available. The heparinase gene can be expressed in these systems, using similar vectors and signal peptides or leader sequences.

Modifications and variations of the present invention will be obvious to those skilled in the art. Such modifications and variations are intended to come within the scope of the following claims.

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 404-815-6508
(B) TELEFAX: 404-815-6555

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1379 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Flavobacterium heparinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CCTTTTGGGA GCAAAGGCAG AACCATCTCC GAACAAAGGC AGAACCAGCC TGTAAACAGA    60
CAGCAATTCA TCCGCTTTCA ACCAAAGTGA AAGCATTTAA TACAATACCA GAATGTCGCA   120
TTTCCCTTTC AGCGTACTTT TTGGGTAAAT AACCAATAAA AACTAAAGAC GGATGAAAAA   180
ACAAATTCTA TATCTGATTG TACTTCAGCA ACTGTTCCTC TGTTCGGCTT ACGCCCAGCA   240
AAAAAAATCC GGTAACATCC CTTACCGGGT AAATGTGCAG GCCGACAGTG CTAAGCAGAA   300
GGCGATTATT GACAACAAAT GGGTGGCAGT AGGCATCAAT AAACCTTATG CATTACAATA   360
```

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 384 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
  (A) ORGANISM: Flavobacterium heparinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Lys Lys Gln Ile Leu Tyr Leu Ile Val Leu Gln Gln Leu Phe Leu
1               5                   10                  15

Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser Gly Asn Ile Pro Tyr Arg
            20                  25                  30

Val Asn Val Gln Ala Asp Ser Ala Lys Gln Lys Ala Ile Ile Asp Asn
        35                  40                  45

Lys Trp Val Ala Val Gly Ile Asn Lys Pro Tyr Ala Leu Gln Tyr Asp
    50                  55                  60

Asp Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr Arg Phe Glu Leu Lys
65                  70                  75                  80

Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala Ala Gly Glu Thr Lys Gly
                85                  90                  95
```

```
Pro Phe Ala Gln Phe Pro Lys Asp Cys Trp Ile Thr Phe Asp Val Ala
    290                 295                 300

Ile Asp Trp Thr Lys Tyr Gly Lys Glu Ala Asn Thr Ile Leu Lys Pro
305                 310                 315                 320

Gly Lys Leu Asp Val Met Met Thr Tyr Thr Lys Asn Lys Lys Pro Gln
                325                 330                 335

Lys Ala His Ile Val Asn Gln Gln Glu Ile Leu Ile Gly Arg Asn Asp
            340                 345                 350

Asp Asp Gly Tyr Tyr Phe Lys Phe Gly Ile Tyr Arg Val Gly Asn Ser
        355                 360                 365

Thr Val Pro Val Thr Tyr Asn Leu Ser Gly Tyr Ser Glu Thr Ala Arg
    370                 375                 380
```

```
Leu Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser Gly Asn Ile Pro
                20                  25                  30
Tyr Arg Val Asn Val Gln Ala Asp Ser Ala Lys Gln Lys Ala Ile
                35          40                  45
Ile Asp Asn Lys Trp Val Ala Val Gly Ile Asn Lys Pro Tyr Ala
                50                  55                  60
Leu Gln Tyr Asp Asp Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr
                65                  70                  75
Arg Phe Glu Leu Lys Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala
                80                  85                  90
Ala Gly Glu Thr Lys Gly Arg Thr Glu Leu Ser Tyr Ser Tyr Ala
                95                  100                 105
Thr Thr Asn Asp Phe Lys Lys Phe Pro Pro Ser Val Tyr Gln Asn
                110                 115                 120
Ala Gln Lys Leu Lys Thr Val Tyr His Tyr Gly Lys Gly Ile Cys
                125                 130                 135
Glu Gln Gly Ser Ser Arg Ser Tyr Thr Phe Ser Val Tyr Ile Pro
                140             145                     150
Ser Ser Phe Pro Asp Asn Ala Thr Thr Ile Phe Ala Gln Trp His
                155                 160                 165
Gly Ala Pro Ser Arg Thr Leu Val Ala Thr Pro Glu Gly Glu Ile
                170                 175                 180
Lys Thr Leu Ser Ile Glu Glu Phe Leu Ala Leu Tyr Asp Arg Met
                185                 190                 195
Ile Phe Lys Lys Asn Ile Ala His Asp Lys Val Glu Lys Lys Asp
                200                 205                 210
Lys Asp Gly Lys Ile Thr Tyr Val Ala Gly Lys Pro Asn Gly Trp
                215                 220                 225
Lys Val Glu Gln Gly Gly Tyr Pro Thr Leu Ala Phe Gly Phe Ser
                230                 235                 240
Lys Gly Tyr Phe Tyr Ile Lys Ala Asn Ser Asp Arg Gln Trp Leu
                245                 250                 255
Thr Asp Lys Ala Asp Arg Asn Asn Ala Asn Pro Glu Asn Ser Glu
                260                 265                 270
Val Met Lys Pro Tyr Ser Ser Glu Tyr Lys Thr Ser Thr Ile Ala
                275                 280                 285
```

9. The nucleic acid molecule of claim 1 wherein the nucleic acid molecule encodes a heparinase having a specific activity different from the specific activity of the heparinase encoded by Sequence No. 1.

10. The nucleic acid molecule of claim 1 in a procaryotic cell other than *F. heparinum* which is capable of expressing the molecule.

11. The nucleic acid molecule of claim 11 in a procaryotic cell cultured under low sulfate conditions which is capable of expressing the molecule.

FIG. 1

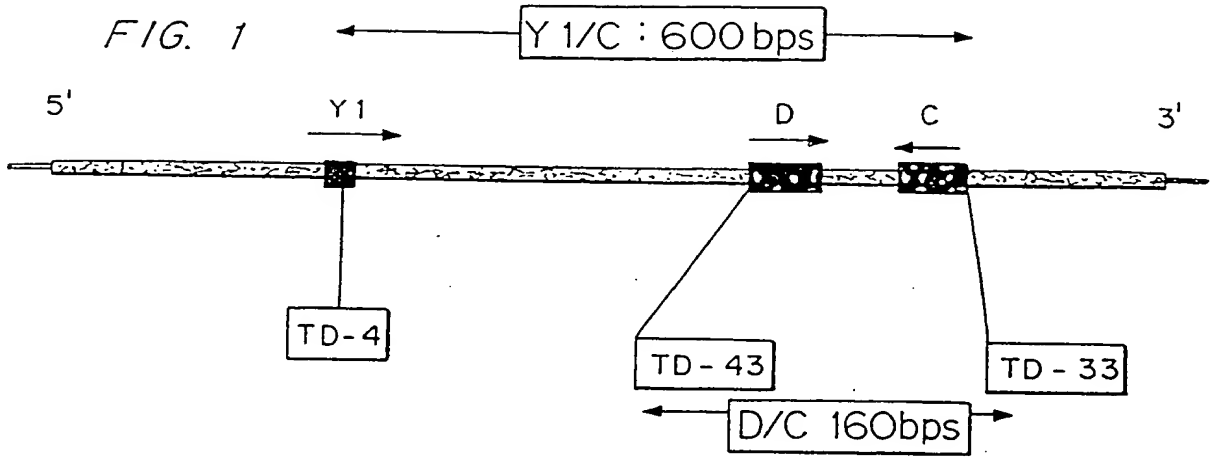
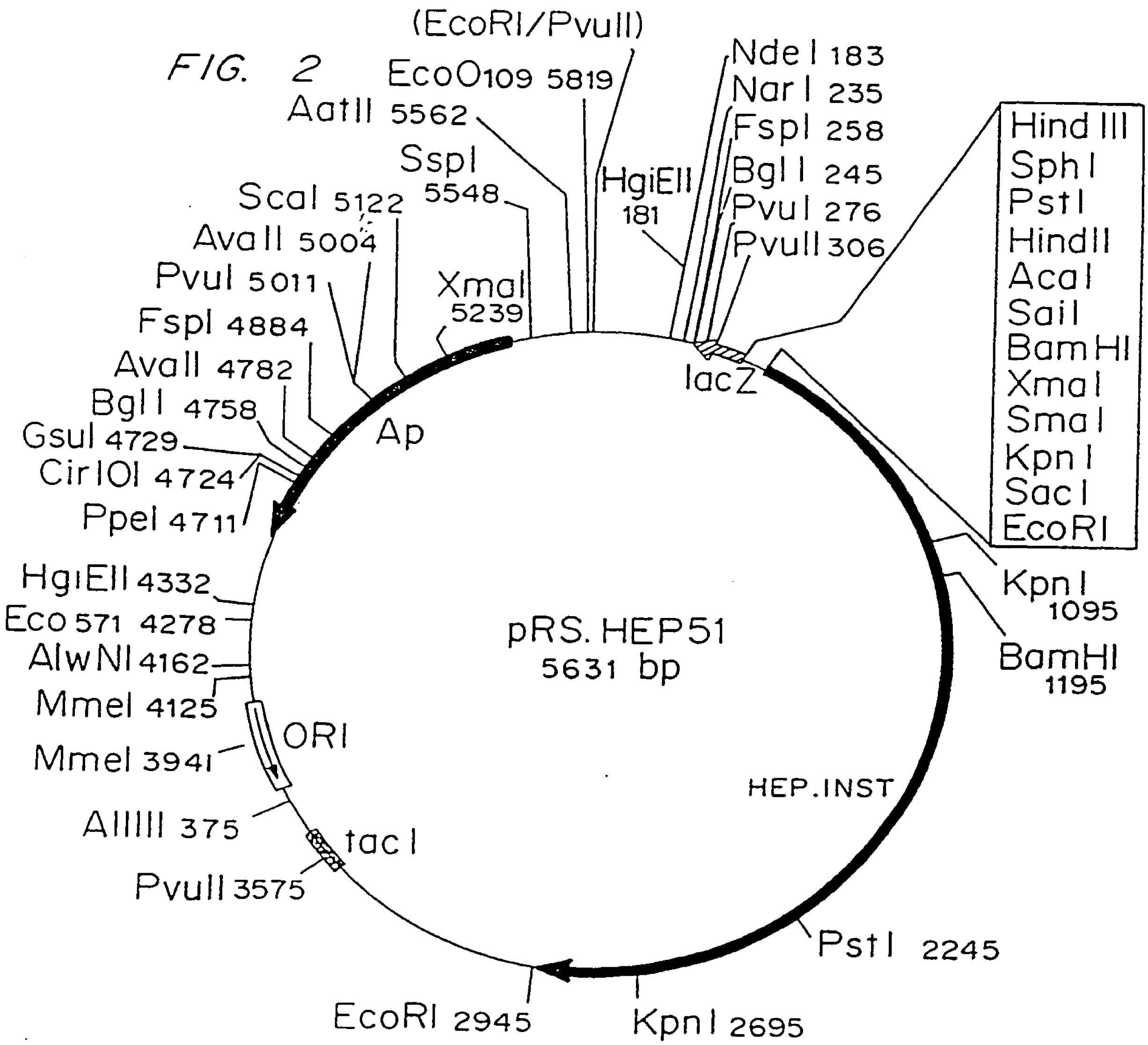

Y 1/C : 600 bps
5'
Y 1
D
C
3'
TD-4
TD-43
TD-33
D/C 160bps

FIG. 2


(EcoRI/PvuII)
EcoO109 5819
AatII 5562
SspI 5548
ScaI 5122
AvaII 5004
PvuI 5011
XmaI 5239
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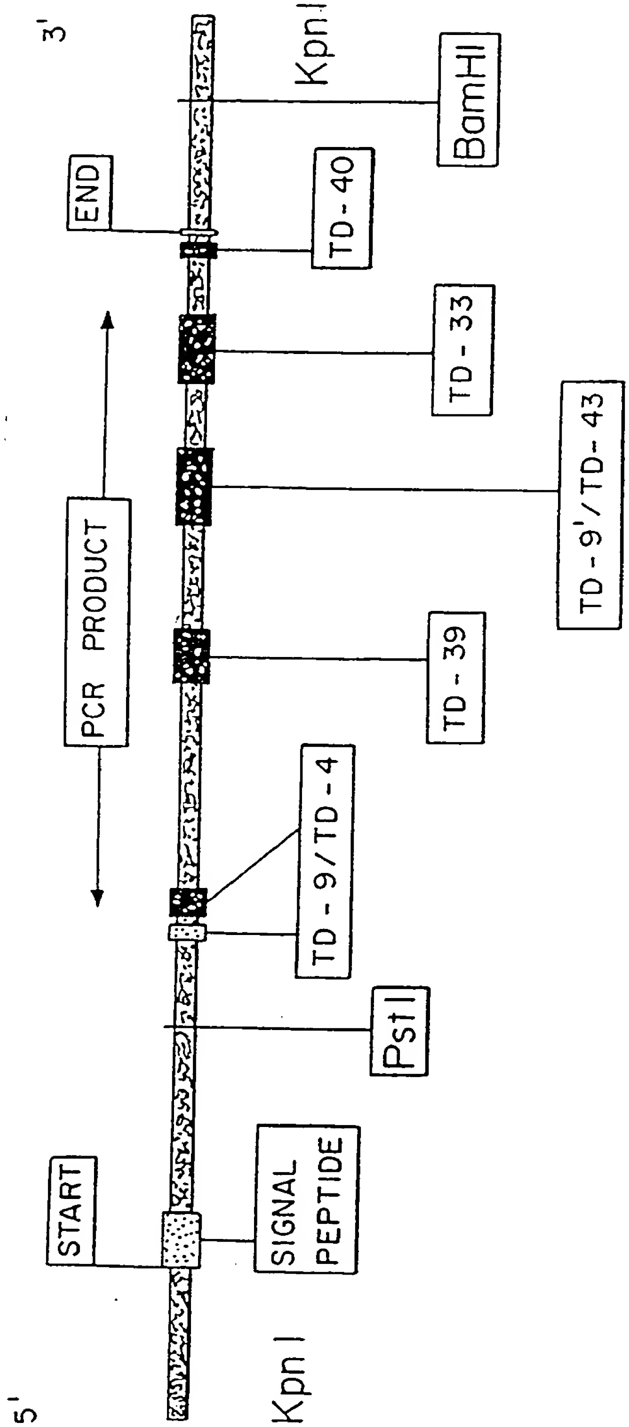
AvaII 4782
BglI 4758
GsuI 4729
Cir101 4724
PpeI 4711
Ap
HgiEII 181
NdeI 183
NarI 235
FspI 258
BglI 245
PvuI 276
PvuII 306
lacZ
Hind III
SphI
PstI
HindII
AcaI
SalI
BamHI
XmaI
SmaI
KpnI
SacI
EcoRI
KpnI 1095
BamHI 1195
HgiEII 4332
Eco571 4278
AlwNI 4162
MmeI 4125
MmeI 3941
ORI
AlIIII 375
tacI
PvuII 3575
pRS.HEP51
5631 bp
HEP.INST
PstI 2245
EcoRI 2945
KpnI 2695

FIG. 3


5'
3'
Kpn I
Kpn I
START
SIGNAL PEPTIDE
Pst I
TD - 9/TD - 4
PCR PRODUCT
TD - 39
TD - 9'/TD- 43
TD - 33
TD - 40
END
BamHI

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/09124

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/60

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,8 912 692 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 28 December 1989 see page 18, line 30 - page 20, line 9	1-11
X	Week 9125, Derwent Publications Ltd., London, GB; AN 91-180925 & JP,A,3 108 486 (SHINGIJUTSU KAIHATSU) 8 May 1991 see abstract	1-11

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
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